Connective Tissue Biomatrix: Its Isolation and Utilization for Long-term Cultures of Normal Rat Hepatocytes

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ABSTRACT A new procedure is introduced for the isolation of connective tissue fibers, called biomatrix, containing a significant portion of the extracellular matrix (basement membrane components and components of the ground substance). Biomatrix isolated from normal rat liver contains >90% of the tissue's collagens and all of the known collagen types, including types I and III and basement membrane collagens. The purified collagenous fibers are associated with noncollagenous acidic proteins (including fibronectins and possibly small amounts of glycosaminoglycans). Procedures are also described for preparing tissue culture substrates with these fibers by either smearing tissue culture dishes with frozen sections or by shredding the biomatrix into small fibrils with a homogenizer. The biomatrix as a substrate has a remarkable ability to sustain normal rat hepatocytes long-term in culture. The hepatocytes, which on tissue culture plastic or on type I collagen gels do not survive more than a few weeks, have been maintained for more than 5 mo in vitro when cultured on biomatrix. These cells cultured on rat liver biomatrix show increased attachment and survival efficiencies, long-term survival (months), and retention of some hepatocyte-specific functions.

Despite numerous advances in cell culture procedures (2, 3, 14, 16, 18, 24, 35), the culture of differentiated epithelial cells, particularly from normal tissues, has remained especially difficult. We have proposed that the shortcomings of cell culture techniques are primarily that cells are isolated from the extracellular matrix and from association with other cell types with which they may be interdependent (30). Culture methods, such as organ culture or the culture of tissue fragments, which retain tissue architecture, preserve the differentiative state of the cells, whereas clonal cell cultures usually undergo a dedifferentiative process (16, 40). Thus, to culture differentiated cells it seems logical to ascertain the critical variables of the tissue matrix and to evolve culture procedures dependent upon them.

In a previous paper, we presented techniques that we refer to as "socio-cell culture techniques" (30) and that are, in essence, attempts to simulate some of the cell-cell relationships of the tissue matrix relevant to epithelial cells. The techniques we described involve the culturing of epithelial cells on substrates of reconstituted basement membrane and in medium supplemented with hormones, serum, and with conditioned medium from feeder layers of primary cultures of fibroblasts. In this article, we present new techniques that have superseded the earlier ones. They include the isolation of connective tissue fibers called biomatrix, representing a significant portion of the in vivo extracellular matrix (basement membranes and ground substance), and techniques using these fibers as substrates for cultures of differentiated cells. Our long-term studies are concerned with the roles of the extracellular matrix and of cell-cell relationships in the differentiation of normal and malignant epithelial cells. In this paper, we present our studies on the use of liver-derived biomatrix as a substrate for normal rat hepatocytes.

MATERIALS AND METHODS

Animals

Adult Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, Wilmington, Mass., and used immediately for purification of type I collagen and/or biomatrix.

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Preparation of Isolated Rat Hepatocytes

Suspensions of isolated rat hepatocytes were prepared by the method of Leffert et al. (21). The cells were washed free of bacterial collagenase with culture medium, diluted, and counted. Cell viability was determined by trypan blue exclusion.

Culture Conditions

MEDIA AND SOLUTIONS: The cells were cultured in minimum essential medium (MEM) or a 1:1 mixture of Dulbecco's modified Eagle's medium (DME) and Ham's F12 supplemented with trace elements (14), HEPES, and with 10% fetal bovine serum (FBS). The medium is referred to as DME/F12 + FBS. All supplies for medium were obtained from Grand Island Biological Co., Grand Island, N. Y. The trace elements were a gift from R. Ham (University of Colorado, Boulder, Colo.). Dulbecco's phosphate-buffered saline (PBS) without magnesium or calcium salts was prepared from reagent grade chemicals from Baker.

PLASTICS: All tissue culture plastics were obtained from Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.

SUBSTRATES: The cells were grown on one of three possible substrates: tissue culture dishes, collagen gels, or rat liver biomatrix. Preparation of the latter two is described below.

Preparation of Rat Liver Biomatrix

After decapitation of a rat, the liver was homogenized gently in a loose-fitting glass Teflon homogenizer, using 10 vol of ice-cold distilled water containing 0.1% sodium azide, and immediately passed through a polyester filter (PeCap HD 7-85, from TETKTO Inc., Elmsford, N. Y.) of 166–168 mesh per inch. The material retained on the polyester was collected with a spatula and then purified as described by Meezan et al. (23) with the modifications described in Fig. 1. All other operations were performed at 4°C. Triplicate aliquots of all filtrates and final retentate were hydrolyzed in 6 N HCl for 24 h at 104°C and analyzed for hydroxyproline content by the procedure of Rojkind and Gonzalez (32). Collagen was estimated from the amount of hydroxyproline, assuming that each polypeptide chain of 100,000 daltons contains 1,000 residues (11).

Morphological Characterization of the Rat Liver Biomatrix

Rat liver biomatrix obtained at different stages of purification was fixed in 2% glutaraldehyde in phosphate buffer, dehydrated with an ethanol series, embedded in Epon, and sectioned for electron microscopy. Freshly prepared fibers were also fixed in buffered formaldehyde, dehydrated with an ethanol series, embedded in paraffin, and sectioned at 5 μm. The sections were stained for reticulin fibers by the method of Gordon and Sweets (12).

After the final stages of purification, rat liver biomatrix was stained in whole mount preparations with ruthenium red by the method of Luft (22) or by periodic acid-Schiff (PAS) staining. Fibronectin analysis was determined by indirect immunofluorescence. Frozen sections, 8 μm each of collagen gels or of biomatrix with or without cells attached, were fixed in 2% paraformaldehyde in 0.1 M PBS and stained with rabbit antichicken fibronectin (a gift of Dr. Shin, Albert Einstein College of Medicine). The sections were secondarily stained with fluorescein-labeled antirabbit antiserum. The fibronectin used as antigen was purified from embryo fibroblast cell cultures by the method of Yamada et al. (42) as modified by Kahn and Shin (personal communication). The stained slides were evaluated with a Zeiss fluorescence microscope. Control slides were stained with rabbit serum from nonimmunized rabbits.

Collagenase Digestion of Purified Rat Liver Biomatrix

Biomatrix (30–50 mg of wet weight) was incubated for 24 h at 20°C with 1 ml of 0.05 M Tris-HCl buffer, pH 7.4, with 0.05 M $CaCl_2$, containing 20 μg of bacterial collagenase (412 U/mg), Worthington Biochemical Corp., Freehold, N. J.). Bacterial collagenase was freed of nonspecific protease activity as described by Peterkofsky and Diegelman (27) and Benya et al. (1). After incubation, the fibers were centrifuged at 3,000 rpm for 30 min and the supernate was transferred to a vial and evaporated to dryness. 1 ml of constant boiling HCl (Pierce Chemical Co., Rockford, Ill.) was added, and the sample was sealed under vacuum. The residue after collagenase digestion was transferred to a second vial and also prepared for acid hydrolysis. Samples were heated at 104°C for 24 h and used for amino acid analysis (9). Corrections were made for recovery of amino acids using norleucine as a standard. No corrections were made for losses after acid hydrolysis. The amount of collagen was estimated from the hydroxyproline content of the sample as described above. The noncollagenous proteins were estimated from the amino acid composition of the sample.

1. Mince tissue and homogenize by hand or in a Waring blender, using 5–10 vol of 0.1% sodium azide solution per gram of tissue. Omit the azide and use only cold distilled water for biomatrix to be used for cell culture substrates. Keep sample at 4°C. Ideal amount of starting material, 100 grams.
2. Immediately filter through polyester into 2- to 4-liter beaker in ice. Wash with more azide solution (water) if necessary.
3. Collect material on top of the polyester mesh and put into a beaker with 1.0% sodium azide (water). Wash polyester mesh thoroughly and add the wash to the beaker.
4. Stir for 1 h at 4°C.
5. Filter through polyester and repeat step *3*, but use 1.0 M NaCl instead of sodium azide (water).
6. Stir for 1 h at 4°C.
7. Collect precipitate by filtration through the polyester mesh. If the solution is very opaque, repeat steps *5–7*. Repeat until the solution is clear.
8. Put the precipitate retained on the filter into a small amount of serum-free medium (~30–50 ml/100 g of starting material) and add 1.0 mg of DNase plus 5 mg of ribonuclease (Sigma Chemical Co.) per 50 ml of serum-free medium.
9. Stir for 1 h at 37°C.
10. Check a sample of the fibers with Acridine Orange (0.3% in distilled water). The nucleic acid contaminants stain an intense orange with this solution. Fibers reasonably free of these contaminants stain a pale salmon color. Repeat steps *8–10* until the fibers are clean of nucleic acid components.
11. Collect precipitate by filtration through polyester and rinse in 1 M NaCl or in serum-free medium for 0.5 h.
12. Collect precipitate by filtration through polyester and put into 100 ml of 1% deoxycholate.*
13. Stir for 1 h at room temperature.
14. Check a sample of the fibers with oil red O.‡ If translucent red globules are present, repeat steps *12–14* until they disappear. The translucent red material appears within 3–5 min.
15. Collect the fibers by filtration through polyester and wash with 250 ml of distilled water. Wash three times (1 h each) and filter between washings.
16. Stir the fibers overnight in PBS or serum-free medium (1× 10 volume) at 4°C.
17. Collect the fibers by filtration and either use immediately for culture or chemical studies or store by freezing at −20°C in serum-free medium plus 10% glycerol.

FIGURE 1 Isolation of biomatrix. *See note added in proof. ‡Oil red O: 0.5% in 60% isopropanol (stock solution). For use: dilute 6 parts stock to 4 parts water, mix on a vortex, and filter.

Analytical Methods

~300 mg of liver was used for acid hydrolysis and hydroxyproline determination by the method of Rojkind and Gonzalez (32) with the modifications described by Ehrinpreis et al[1]. For carbohydrate analysis, samples of fibers of 20–30 mg of wet weight were hydrolyzed for 3 h at 104°C with 2 ml of 2 N HCl. Excess HCl was evaporated to dryness in a flash evaporator, and the sample was dissolved in 2 ml of distilled water. Aliquots (0.5–1.0 ml) were used for hexose analysis with anthrone as described by Seifter et al. (37). Glucose was used as a standard, and the results are expressed as glucose equivalents per milligram of biomatrix. Protein determinations were done according to the method of Lowry et al. (20).

Uronic acid determination was performed on 1 g of fresh liver or 100 mg of biomatrix by a modification (19) of the carbazole reaction (5, 7). Liver or biomatrix was first incubated for 24 h at 4°C with 0.1 N NaOH, neutralized with

[1] Ehrinpreis, M., M. Giambrone, and M. Rojkind. 1980. Liver proline oxidase activity and collagen synthesis in rats with cirrhosis induced by carbon tetrachloride. *Biochim. Biophys. Acta.* 629:184–193.

0.1 N HCl, and digested for 24 h with pronase (19). Protein was precipitated with TCA (final concentration of 10%) and removed by centrifugation. The supernate was dialyzed exhaustively against distilled water, and aliquots were used for uronic acid determination. Qualitative determination of the presence of glycoproteins and glycosaminoglycans was done by ruthenium red (22) staining.

Preparation of the Cell Culture Substrates

COLLAGEN GEL RAFTS: Collagen gel rafts were prepared as described in the review by Reid and Rojkind (30).

BIOMATRIX: In preparing biomatrix for cell culture substrates, we omitted sodium azide in the isolation procedures because it was difficult to remove from the matrix and was toxic to the cells. Meticulousness in the isolation of biomatrix was required to prevent carry over of any toxic substance, such as deoxycholate. To ensure quality control of the preparation, we examined the fibrous matrix after each step to be sure that the solubilization was complete. For example, after treatment with nucleases, a sample of the fibers was stained with acridine orange. Treatment with nucleases was continued until the biomatrix stained a pale salmon color with acridine orange. After solubilization of the lipids with sodium deoxycholate, a sample of the fibers was soaked in oil red 0. If translucent red globules indicating the presence of residual lipid contaminants were found, deoxycholate treatment was continued. Particularly important were the final rinses in PBS and serum-free medium, which were essential for elimination of deoxycholate.

To make culture substrates, the best results were obtained by embedding the biomatrix into polyvinyl pyrrolidone, Tissue Tek (Lab-Tek Products, Div. Miles Laboratories Inc., Naperville, Ill.; which prevents ice-crystal formation), and freezing it at −20°C. 5- to 10-μm sections were cut on a cryostat and smeared over tissue culture plates with small camel hair brushes. Tissue Tek reduced attachment efficiency of the cells on the biomatrix. Therefore, the plates on which the Tissue Tek was used were thoroughly rinsed with three to four changes of PBS and soaked overnight with serum-free medium before use of the plates for culture experiments. An alternative to the above was to homogenize the biomatrix with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, N. Y.) until it was shredded into short fibrils of 1–3 mm in length. The homogenization was done in PBS or serum-free medium maintained at 4°C and with 10- to 15-s bursts of the homogenizer. Cells were attached to the fibrils and either allowed to sit on the bottom of the dish or kept in suspension by the use of a sterilized magnetic stirrer. With this method the cells do not have to be detached in order to subculture the plate. A portion of the fibrils with attached cells can be transferred to a new dish with more fibrils.

Sterilization of the biomatrix fibrils or of the plates smeared with biomatrix was done by irradiation with 10,000 rads of cobalt gamma radiation. Although sterilization of the substrates could also be effected by 5,000 rads of gamma irradiation or by UV irradiation, the sterilization procedures were occasionally inadequate for control of mold spores. Therefore, the high-dosage gamma irradiation was adopted exclusively.

Evaluation of the Cultures

MORPHOLOGIC STUDIES: Suspensions of isolated rat hepatocytes were added to either tissue culture dishes, type-I collagen gel rafts or rat liver biomatrix. Cultures were maintained for up to 5–6 mo, with the media being changed once weekly. Photographs were taken at regular intervals with a Nikon inverted phase microscope. For histological studies, cell cultures were fixed in methanol or with buffered formaldehyde, dehydrated, and embedded in paraffin. 8-μm sections were stained with hematoxylin/eosin.

ATTACHMENT EFFICIENCY: Suspensions of rat hepatocytes at varying densities were plated onto one of the three substrates. The cultures were provided with a medium of MEM or DME/F12 + FBS and incubated at 37°C in an incubator flushed with 95% air and 5% CO_2. The cultures were maintained for 24 h after which the plates were rinsed twice with PBS, and the attached cells were removed with 0.1% collagenase in serum-free medium (type I, Sigma Chemical Co., St. Louis, Mo.). The number of viable cells was counted and analyzed by trypan blue exclusion.

LONG-TERM SURVIVAL EFFICIENCIES AND THE DURATION OF THE CULTURES: Varying densities of rat hepatocytes were added to 35- or 60-mm dishes with one of the possible substrates. The cultures were provided with a medium of MEM or DME/F12 + FBS and incubated at 37°C in an incubator flushed with 95% air and 5% CO_2. The cultures were maintained for 3–4 wk, after which the plates were rinsed and the cells detached with collagenase (type I, Sigma Chemical Co.). The number of viable cells was ascertained by trypan blue exclusion. The potential duration of the cultures was estimated by plating 10^5–10^6 cells on one of the various substrates in 35- and 60-mm dishes and maintaining the cultures with weekly changes of medium for as long as the cells seemed viable. If the cultures seemed to be dying, the experiment was terminated, and cells were detached by enzymatic treatment and evaluated for viability. At regular intervals, cultures that appeared healthy were terminated to ascertain the number of viable cells and the presence of hepatocyte-specific markers. Some of the long-term cultures were checked for mycoplasma according to the method of Chen (4).

Albumin and Ligandin Markers

Hepatocytes at 10^5–10^6 cells per 35- or 60-mm dishes were seeded and cultured as described above. At varying intervals, the cultures were terminated and the presence of albumin or ligandin in the cells was ascertained by indirect immunofluorescence. The cells were detached from the substrates, rinsed with PBS, fixed in ice-cold acetone, stained with antirat albumin or antirat ligandin (both made in goats), and secondarily stained with fluorescein-labeled rabbit antigoat immunoglobulin. The stained slides were evaluated with a Zeiss fluorescence microscope. Control slides were stained with rabbit serum from nonimmunized rabbits.

In a sampling of the cultures, albumin synthesis was evaluated by incubating the cultures with labeled amino acids (New England Nuclear, Boston, Mass.). The cultures were then collected, homogenized, and boiled in 2% SDS with mercaptoethanol. The samples were run on 7.5% SDS-polyacrylamide gels. Fluorography for radioactivity in the area of the albumin standard band indicated the presence of newly synthesized albumin.

RESULTS

Isolation and Characterization of Rat Liver Biomatrix

A procedure given in Fig. 1 was developed for the isolation of fibers from the extracellular matrix. The method includes sequential solubilization of the tissue components by water, sodium chloride buffers, nucleases, and deoxycholate solutions. With this method, a fibrous matrix was isolated containing >85% of the tissue collagens and whatever noncollagenous proteins and small amounts of glycosaminoglycans that are normally in close association with the collagens. As shown in Table I, the biomatrix contains primarily two components. After digestion with bacterial collagenase, the amino acid composition of the solubilized material is similar to that expected for collagen. The collagenase-insoluble residue has an

TABLE I

Amino Acid Composition of Collagenase-soluble and Collagenase-resistant Proteins of the Liver *

Amino acid	Collagenase soluble	Collagenase resistant
	Residues/ 1,000 amino acid residues	
4-Hydroxyproline	78	0
Aspartic Acid	57	117
Threonine‡	23	44
Serine‡	19	31
Glutamic Acid	71	117
Proline	96	57
Glycine	301	131
Alanine	102	78
1/2 Cystine‡	trace	31
Valine	41	68
Methionine§	7	7
Isoleucine	26	57
Leucine	41	71
Tyrosine‡	3	13
Phenylalanine	22	42
Hydroxylysine	10	0
Lysine	46	77
Histidine	14	21
Arginine	43	38

* Average of two preparations.
‡ Not corrected for losses after acid hydrolysis.
§ Determined as methionine and methionine sulfoxides.

amino acid composition similar to that of fibronectin (26, 38, 39, 42) or the acidic glycoproteins associated with elastin (33) or collagen (38). Although we have not determined at present the nature of the noncollagenous proteins associated with the collagens, the presence of fibronectin was established after staining the biomatrix with antiserum to chicken fibronectin as shown in Fig. 5*A*.

The presence of glycosaminoglycans is suggested but has not been firmly established. The biomatrix fibers stained strongly with ruthenium red and PAS stains. However, uronic acid determinations by the carbazole method, although positive, were subsequently found to be biased by the presence of other hexoses in large amounts. Studies to clarify this matter are under way.

A comparison of collagen gels derived from rat-tail tendons and skin with the biomatrix fibers was made using histochemical and immunofluorescence procedures. The collagen gels prepared from rat-tail collagen contained no type-III collagen (silver staining), fibronectin (indirect immunofluorescence), or PAS, or ruthenium red–positive material. The gels prepared from skin extracts had trace amounts of type-III collagen, fibronectin, and PAS, and ruthenium red-positive material. In contrast, the biomatrix was strongly positive for all of these components.

The collagens from rat liver biomatrix were isolated after pepsin digestion (31). As shown in Tables I and II, the biomatrix contains types I and III and basement membrane collagens. The collagens present in the biomatrix were solubilized by pepsin digestion, and the collagen alpha chains recovered quantitatively indicating that treatment of the tissue with 1% deoxycholate did not denature the collagen fibers. Denatured collagen is readily degraded after pepsin treatment (31). Hydroxyproline analysis of the biomatrix as compared with that of the liver revealed that at least 80% of the collagen present initially in the liver was recovered in the biomatrix. The recovery of collagen was increased to 95% by washing the polyester after each step and retrieving the small amounts of fine collagen fibrils entrapped in the polyester filter. The nature of the collagen lost has not been determined. The matrix contains equal amounts of type I and III collagens, a small amount of A + B components (~6%), and ~10% undefined material (Table II). This latter corresponds to the basement membrane collagen (type IV) and to small amounts of minor, undefined components normally present in the liver. Further characterization of the collagenous components of the biomatrix has been presented elsewhere.[2]

TABLE II

Composition of the Biomatrix Derived from Rat Liver

	Collagens	Types of collagen	Noncollagenous proteins	Carbohydrates
	(mg/100 mg protein)	*(% of total collagen)*	*(mg/100 mg protein)*	*(μmol glucose equiv./100 mg protein)*
Prep I	63.7	Type I, 43% Type III, 42.7% Basement membrane collagens (A + B), 6.2% * Undefined, 10.9%	36.2	9.0
Prep II	60.9	As above	39.1	10.0

Proportion of the liver recoverable as biomatrix (given as [amount in biomatrix]/[amount in liver] × 100 = %): Wet wt (g), 1.14%; the yield of biomatrix by weight is the average of 50 normal rat livers. Total collagen (mg), 95%; the % yield of collagen in the biomatrix is the average of 5 normal rat livers. Total protein (mg), 0.87%; noncollagenous proteins (mg), 0.35%; the % yield of total protein and noncollagenous proteins is the average of two normal rat livers.

* *Undefined* represents a mixture of type-IV collagen and other minor components not yet characterized.

Morphological Studies

In Fig. 2*A* is seen freshly prepared rat liver biomatrix. The heterogeneous nature of the biomatrix is apparent even from the macroscopic appearance of the material. Histochemical studies indicated that the filamentous portions (*C*) were primarily collagenous in composition with little ruthenium red–positive material. Other areas with golden-colored, globular material (*G*) stained intensely with ruthenium red and PAS stains. Fibroblasts and endothelial cells attached preferentially to the filamentous (*C*) regions, whereas hepatocytes attached to regions enriched in the ruthenium red–positive material (*G*). In Fig. 2*B* is seen a 48-h culture in which hepatocytes are attached to the *G*-region of the biomatrix and are noticeably absent on the surrounding tissue culture plastic. In Fig. 3*A* and *B* are hepatocyte cultures maintained for 4 mo. The cells have spread over the matrix and have prominent "chicken-wire-like" channels separating the cells. Although mitotic figures were occasionally observed, the cultures either grew slowly or not at all. Subculturing was rarely required for up to 5 mo of culture on the biomatrix.

Hematoxylin- and eosin-stained preparations (Fig. 4*A* and *B*) of hepatocyte cultures after 40 d on biomatrix indicate a large number of viable cells interspersed with a network of channels. The opaque ovals are dead cells that also attached to the fibers. These dead cells were seen from the earliest stages of the cultures and are assumed to derive from the procedures used to isolate the hepatocytes from liver. Large vacuolar sites within the cells are of unknown composition and await further characterization by ultrastructural and histochemical studies.

Attachment Efficiency/Survival Efficiency/Growth Studies

The attachment efficiency of the hepatocytes plated onto the various substrates is shown in Table III. As previously demonstrated by others (24), the hepatocytes have a low attachment and survival efficiency on tissue culture plastic but a dramatically enhanced attachment and survival efficiency on collagen gel rafts. Of the substrates tested, the biomatrix proved superior in attachment efficiency, survival efficiency, and duration of the cultures. Attachment efficiencies on the 35-mm tissue culture dishes coated with biomatrix of >70% were consistently observed at all seeding densities up to 10^5. However, above 10^5, saturation binding phenomenon was seen. Increasing numbers of cells seeded above 10^5 cells did not significantly increase the number of cells bound.

The differences in the substrates became increasingly apparent in the studies on the duration of the cultures. The number of cells on plastic declined steadily until by 2–3 wk almost all the cultures contained no viable cells. Similar to the findings of others (24), the cultures of hepatocytes maintained on col-

[2] Rojkind et al. *J Biol. Chem.* In press.

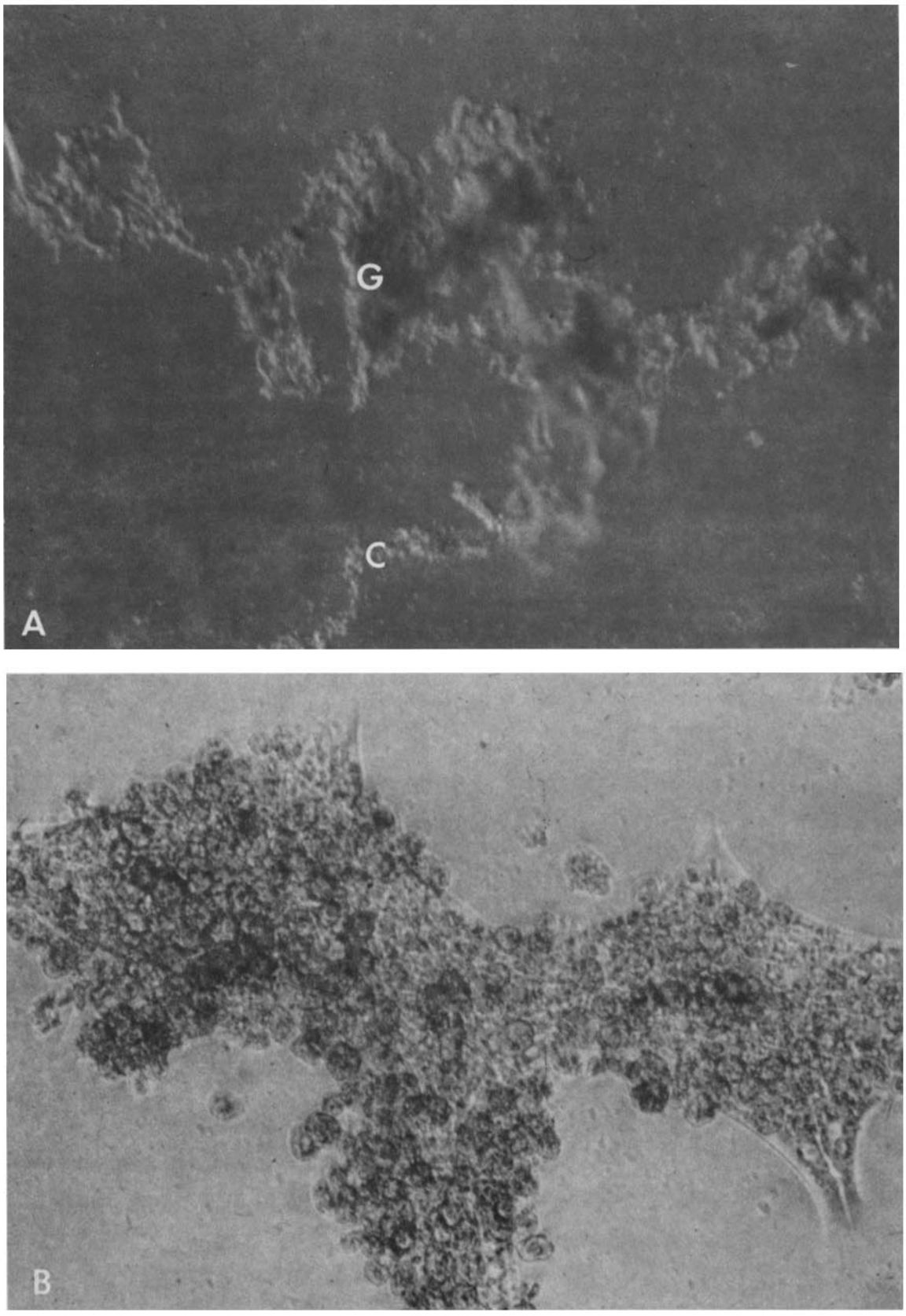

FIGURE 2 Phase microscopy of rat liver biomatrix and of rat hepatocytes on the biomatrix. (*A*) Biomatrix alone. Even by phase microscopy, the biomatrix appears heterogeneous. In some areas it was observed to be filamentous and was found to be primarily collagenous (*C*). In others, the filamentous material was associated with golden globular material (*G*) found to be mixtures of collagens and carbohydrate-rich proteins. Fibroblasts and endothelial cells attached more readily to the filamentous regions; the hepatocytes attached preferentially to the carbohydrate-rich areas. (*B*) 2-d culture of rat hepatocytes on rat liver biomatrix. The hepatocytes attached to the biomatrix and not to the surrounding plastic. Around the edges of aggregates of cells on the matrix can be seen cells that are beginning to spread. × 260.

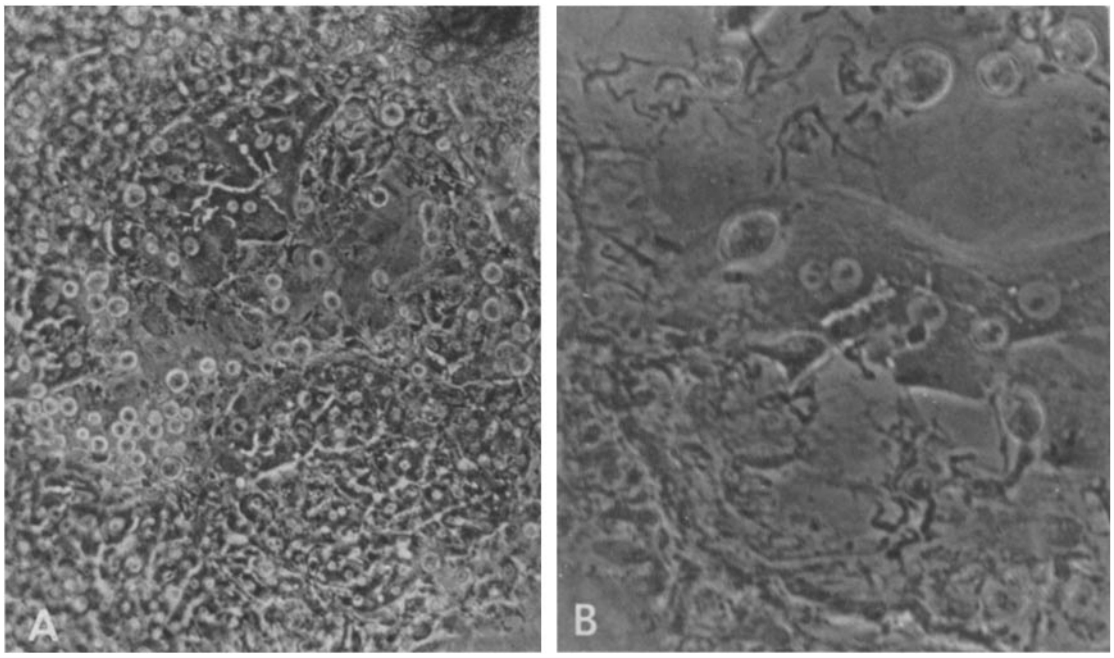

FIGURE 3 Phase microscopy of long-term cultures of rat hepatocytes. The two cultures (*A* and *B*) shown are more than 4 mo old. The cells were plated onto rat liver biomatrix by methods described in Materials and Methods. The hepatocytes have spread over the biomatrix and occasionally are observed with mitotic figures (not seen in these photographs). However, the amount of growth was quite limited. The cultures, which were typical, never required subculturing for almost 5 mo. "Chicken-wire-like" channels are prominent between the cells throughout the cultures. *A*, × 230; *B*, × 260.

lagen gel rafts lasted, on the average, 4–5 wk. After 3 wk, 40–50% of those that had attached and were alive on day 1 were still viable. On the biomatrix, the majority of the cells that were alive 24 h after plating survived for a number of months. In various studies, the cells have been maintained for >5 mo. Growth of the hepatocytes on the biomatrix has been infrequently observed. Occasionally, clones of proliferating hepatocytes were seen, but these have been the exception rather than the rule. Studies with [^{3}H]thymidine and growth curves have not been attempted. Whether or not the hepatocytes can grow on the biomatrix is unclear at this time.

Differentiated Functions

Qualitative studies using indirect immunofluorescence have shown that cells contained albumin and ligandin for as long as the cultures were maintained. In Fig. 5 *B* is a photograph of cells stained by indirect immunofluorescence for ligandin. In Table IV are presented the qualitative data on cultures maintained for >3 mo. Albumin synthesis was confirmed on day-15 cultures by incubating them with labeled amino acids, homogenizing the cells, and running them on SDS gels. Fluorography of the radioactivity in the area of the albumin standard band indicated the presence of newly synthesized albumin material. Quantitative studies of bilirubin conjugation and of the presence of other hepatocyte-specific markers have been submitted elsewhere (29). In brief, the cells after 6–8 wk on biomatrix contained all reactions involved in bilirubin production and conjugation. They were positive for glutathione-S-transferase, glutamic pyruvic transaminase (GPT), and glutamic oxaloacetic transaminase (GOT), and for the ability to metabolize azo-dye carcinogens.

DISCUSSION

We have developed procedures by which one can isolate a portion of the extracellular matrix and use it as a substrate for long-term cultures of normal rat hepatocytes. The use of individual components of the extracellular matrix for culturing cells has long existed. Investigators have used plates coated with collagen in a variety of forms (3, 8, 25) and, more recently, fibronectins (41) for primary cultures of differentiated epithelial cells. The innovation described is the use of a substrate that is a complex mixture of some of the components known to be in the extracellular matrix. The method of isolation reflects the realization of a need for multiple components. As modified from the original procedure of Meezan et al. (23), the method involves a series of solubilizations to eliminate undesired components and repeated selection for unsolubilized fibers longer than 80 μm. The solubilizations eliminate DNA, RNA, lipids, and components solubilized in water or low-ionic-strength salt buffers. The advantages are that the procedures select for native collagen fibers and their associated noncollagenous proteins and carbohydrates, components known to be in basement membranes. As indicated in Tables I and II, the biomatrix isolated from rat liver is composed of all the known liver collagens, some glycoproteins including fibronectins, and possibly some glycosaminoglycans. Further characterization of the noncollagenous proteins and of the possible presence of glycosaminoglycans is currently under way. More extensive characterization of the collagenous components has been presented elsewhere.[2]

Use of the biomatrix as a cell culture substrate indicates that it is substantially better than other substrates currently in use for hepatocytes (24). The attachment and survival efficiencies as indicated in Table III are dramatically improved. The potential lifespan of the cultures using biomatrix is not known. Some cultures have been maintained for >5 mo. The presence of viable and functional hepatocytes has been confirmed by qualitative evaluations of the presence of ligandin and albumin (Table IV and Fig. 5 *B*).

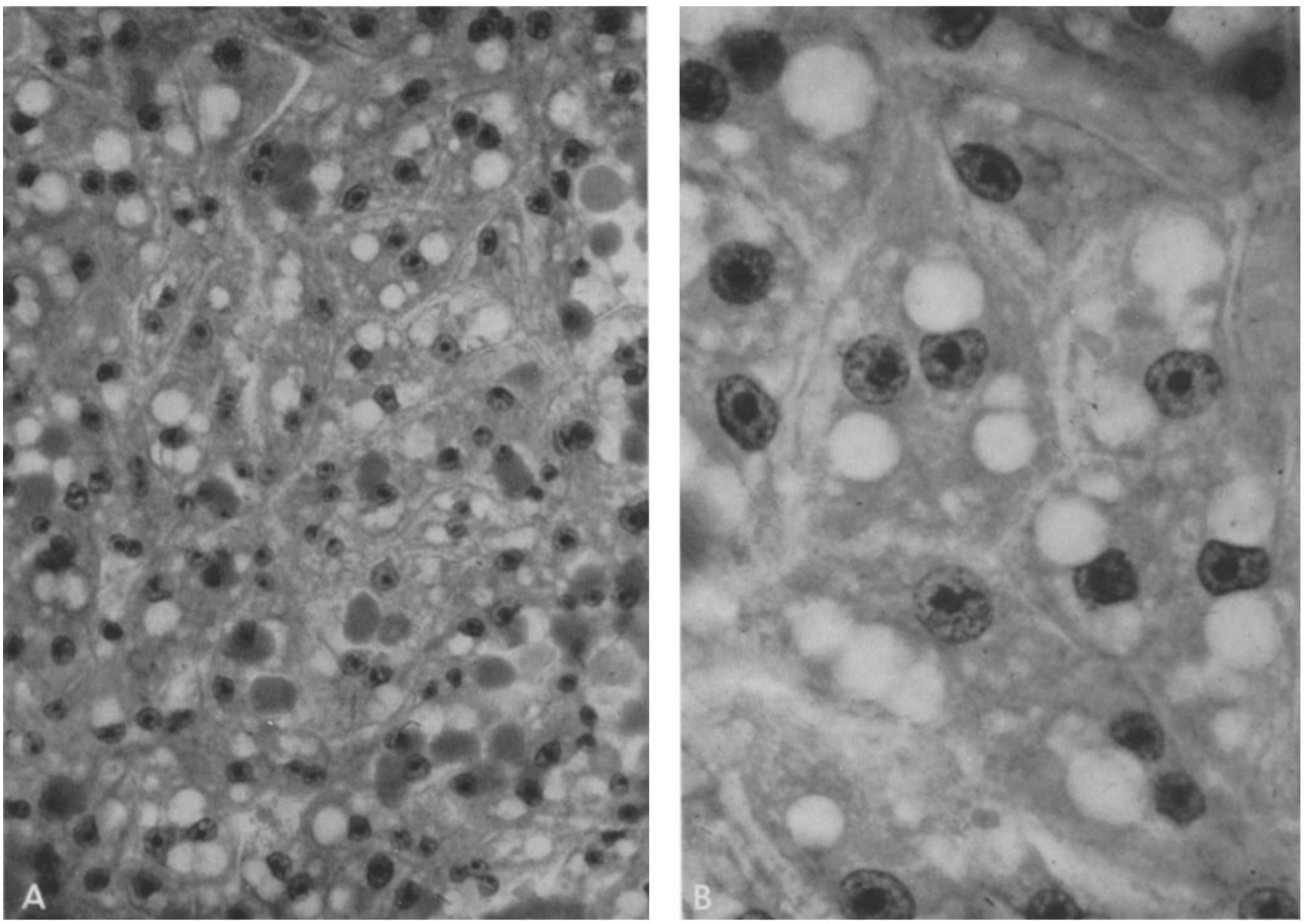

FIGURE 4 Histology of cultures of rat hepatocytes on biomatrix. Hematoxylin- and eosin-stained cultures of hepatocytes cultured on biomatrix smeared onto coverslips. The cultures are 40 d old. A network of channels interspersed with cells and acidophilic ovals is seen. The acidophilic ovals are dead cells thought to have been killed in the procedures for isolating the hepatocytes from the liver. In subculturing the plates, these dead cells do not detach with collagenase treatment. The live cells contain nuclei with prominent nucleoli; the cytoplasm contains vacuoles of unknown composition. *A*, × 200; *B*, × 440.

TABLE III

Attachment and Survival Properties of Rat Hepatocytes on Various Substrates

Property	Seeding density	Substrates: Plastic	Collagen gels	Biomatrix
		%	%	%
Attachment efficiency	10^4	25	30	85
	10^5	30	45	77
	10^6	55	70	10
Survival efficiency of the attached cells at 3 wk	10^4	0	≅50*	>95
	10^5	0	≅50	>95
	10^6	0	≅50	>95
Duration of the cultures	10^4–10^6	1–2 wk	4–5wk	>5mo

* The % of viable cells on collagen gels was quite variable, ranging from 35 to 60%. On the average, ~50% of the cells were still alive after 3 wk.

Whether or not the hepatocytes can grow on biomatrix is unknown, because the investigations into the growth of the cultures are incomplete and inconclusive. In related studies, however, differentiated prostatic tumor cells and Syrian hamster insulinoma cells did grow on biomatrix.[3] Thus, it seems that growth of some cell types is feasible under these new substrate conditions.

The need for collagenous components to enhance survival of normal cells has been strongly implicated in other studies. Recently, Murray et al. (25) have shown that adult guinea pig skin epidermal cells attach more efficiently to purified basement membrane collagen. Michalopoulos and Pitot (24) have used type-I collagen gels to maintain functional rat liver cultures for ~1 mo. In analogous investigations, Emerman and Pitelka (8) used the floating collagen gels to maintain primary cultures of mouse mammary epithelium for 3–4 wk. Our studies corroborate these investigations and suggest that the substrate requirements, as are the hormone requirements (2, 35) of cells in culture, are multiple and complex. Thus, long-term survival and retention of differentiated functions by cells in vitro are dependent upon both extracellular matrix components and mixtures of hormones specific for each cell type. It seems

[3] Reid, L., N. Minato, and M. Rojkind. 1980. Human prostatic cell in culture and in conditioned animals. *In* Male Accessory Sex Organs. E. Spring-Mills and E. Hafez, editors. Elsevier/North Holland Biomedical Press, 617–640. Reid, L., S. Mackensen, Z. Gatmaitan, and B. Morrow. Culture of differentiated cell on substrates of basement membrane material. *In* ICN Symposium on Control of Cellular Division and Development, March 1980. In press.

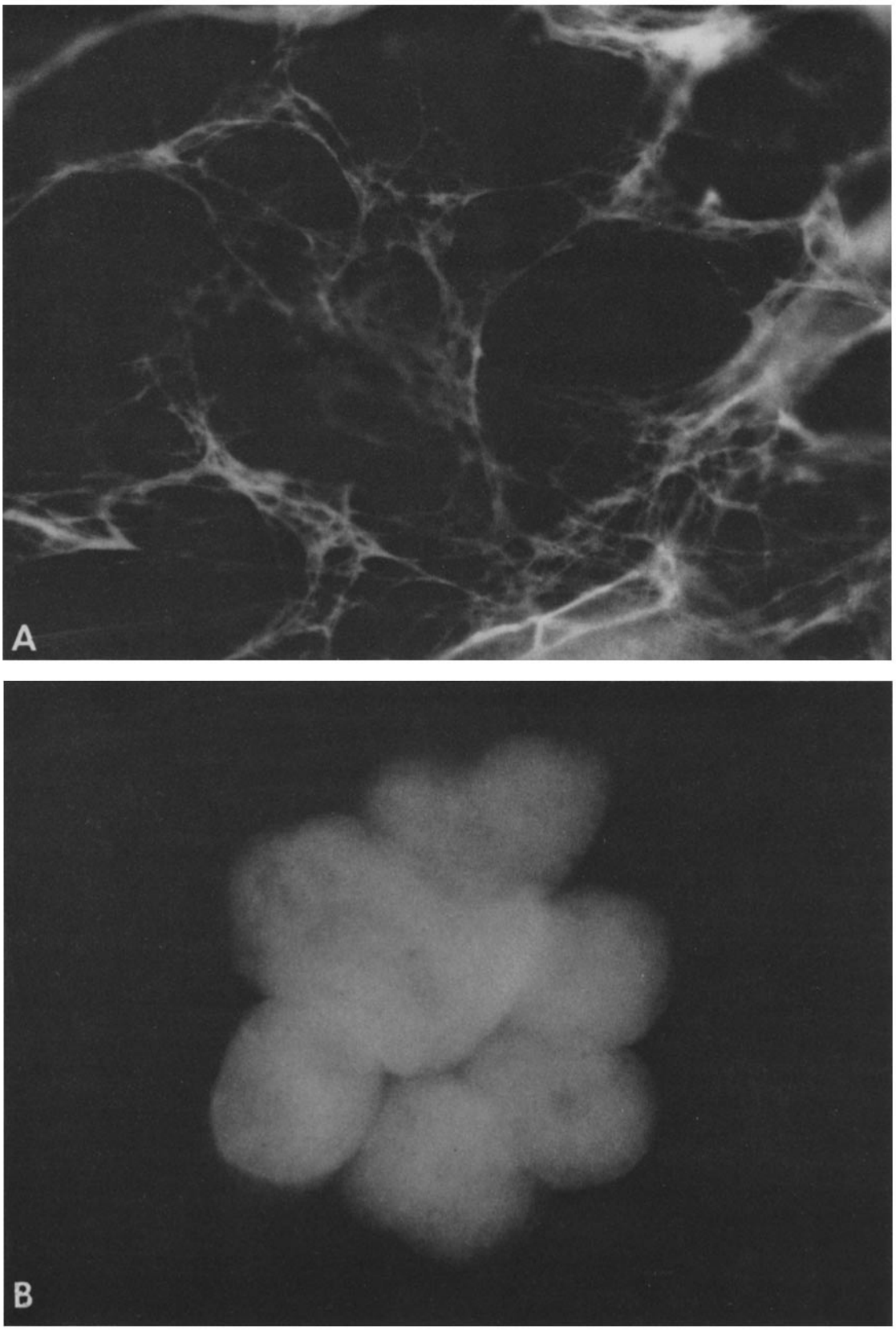

FIGURE 5 Immunofluorescence studies of biomatrix and hepatocytes on the biomatrix. (*A*) Indirect immunofluorescence for fibronectin on rat liver biomatrix. The procedures used are given in the Materials and Methods. The biomatrix, in contrast to the collagen gels from rat tail tendons and from skin extracts, stained intensely by antifibronectin serum. (*B*) Indirect immunofluorescence for ligandin in rat hepatocytes cultured on rat liver biomatrix for more than 2 mo. The cells stained equally well with antiserum directed against albumin. *A*, × 260; *B*, × 570.

TABLE IV

Ligandin and Albumin Markers in Rat Hepatocytes Cultured on Biomatrix

Conditions	Ligandin*	Albumin‡
Whole liver (in vivo)	+	+
Freshly isolated hepatocytes	+	+
Hepatocytes cultured on rat liver biomatrix		
5 d	+	+
15 d‡	+	+
30 d	+	+
60 d	+	+
100 d	+	+

* Evaluated by indirect immunofluorescence as described in Materials and Methods.

‡ Albumin synthesis confirmed as described in Materials and Methods.

apparent that detailed investigations are required to elucidate the relevance of each of the components of the biomatrix or of other extracts of basement membrane material to the survival, growth, and differentiation of cells. The dependence of epithelial cells on basement membrane components is a reflection of their dependence on the epithelial-mesenchymal interaction that has been studied increasingly in a variety of systems (6, 10, 13, 15, 17, 28, 34, 36). In addition to the basement membrane components, Green and his colleagues (13) and Hata and Slavkin (15) have found that the mesenchymal cells produce a soluble product that is present in conditioned medium from cultures of mesenchymal cells and that is important in the regulation of growth or differentiation of the epithelium. Thus, to establish normal cells in culture with retention of their cell-specific markers will require techniques reflecting a synthesis of the many complex variables now known to influence cells in vitro (2, 13, 14, 16, 18, 35).

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Note Added in Proof: In subsequent studies (Reid et al., manuscript in preparation), better delipidation methods were tried and adopted. The detergents were found to leave toxic residues on the matrix. For some tissues with significant lipid deposits, the length of treatment with detergents necessary to eliminate the lipids produce a biomatrix too toxic for cell cultures. Delipidation with butanol/ether mixtures has proved a viable alternative. Butanol/ether solutions at 40:60 ratios can be layered over an equal volume of distilled water. The biomatrix is vortexed in this mixture every 5 min for 0.5–1 h.

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